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19 ABSTRACT (Continue on reverse if necessary and identify by block number)

We have resolved and analyzed in vitro 10 of the 13 separate molecular reactions and pathways controlling metamorphosis of *Haliotis* larvae (in response to exogenous signal molecules) that we first identified in vivo. Two different chemosensory receptors and receptor-dependent signal transduction cascades regulating metamorphosis have now been characterized on highly purified cilia from the larval epithelium. The receptors, a receptor-regulated G protein, and proteins phosphorylated by receptor-regulated protein kinases A and C have been labeled in vitro, and the latter three labeled proteins have been purified.

Three additional proteins have been characterized, and shown to be developmentally regulated in the metamorphosis of *Haliotis* larvae. The cDNA for one of these, a novel serine protease, has been cloned and sequenced. Deductions about the structure and function of the enzyme based on the nucleic acid sequence analysis have been confirmed by measurements of the catalytic properties of the enzyme.

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PROGRESS REPORT ON CONTRACT N00014-87-K-0762; R&T CODE 4412027

PRINCIPAL INVESTIGATOR: Daniel E. Morse

CONTRACTOR: University of California
Santa Barbara, CA

CONTRACT TITLE: Molecular Mechanisms of Chemosensory Receptors, Signal Transducers, and the Activation of Gene Expression Controlling Establishment of a Marine Symbiosis

START DATE: August 1, 1987 (Year 1)
August 1, 1988 (Year 2)

RESEARCH OBJECTIVE:

(1) To characterize the molecular mechanisms by which marine invertebrate larval chemosensory receptors and their associated signal transducers regulate surface attachment and metamorphosis of the larvae in response to chemical signals from the environment; and (2) To characterize the molecular mechanisms regulating the activation of specific gene expression in the developmentally arrested marine invertebrate larva, in response to chemical inducers of metamorphosis.

PROGRESS (Year 2):

Objective 1: Chemosensory receptors and signal transducers:

Our major accomplishment toward this objective this year has been the resolution and analysis in vitro of 10 of the 13 separate molecular reactions and pathways controlling metamorphosis in response to exogenous signal molecules that we had originally deduced from experiments in vivo. (The in vivo observations were reported in last year's report, and published in Proc. Natl. Acad. Sci. 84:1867-1870, by Baxter and Morse.) We have now isolated the chemosensory receptors and receptor-associated signal transducers, functionally intact, in highly purified cilia obtained from the cephalic epithelium of the *Haliotis rufescens* (abalone) larvae. These cilia have been shown in vitro to contain elements of the Morphogenetic Pathway, including: a GABA receptor-dependent activation of a cyclic AMP-controlled protein kinase A (PKA), leading to the receptor-dependent phosphorylation of a unique 25 kD endogenous target protein. The cilia also contain most if not all of the elements of the Regulatory Pathway, including the entire cascade of: lysine receptor-dependent activation of a tightly coupled G protein; G protein-dependent activation of a diacylglycerol-stimulated, calcium-stimulated protein kinase C (PKC); which in turn phosphorylates a unique 130 kD protein within the cilia.

These results with the *Haliotis* larvae represent the first eukaryotic chemosensory system in which the entire sequence of receptor-dependent G protein and protein kinase control has been resolved in vitro. One manuscript reporting these findings is now in press, and another is in preparation.

We have succeeded in specifically labeling the GABA receptor, the lysine receptor, the G protein, and the 25 kD and 130 kD target proteins that are phosphorylated by PKA and PKC under the control of the two chemosensory receptors, and recently have purified the latter 3 labeled proteins. We now are attempting to clone and sequence the genes encoding these chemosensory receptors and signal transducers, to further characterize their structures and mechanisms of action. In one approach, we have purified mRNA from the isolated cilia, and now are probing this mRNA and attempting to drive PCR-amplification to enrich the desired sequences.

Objective 2: Mechanisms of morphogenetic activation of specific gene expression in the developmentally arrested larva:

Three additional proteins have been characterized and shown to be under developmental regulation, with evidence for activation of specific gene expression in metamorphosis and early growth in the transition of *Haliotis rufescens* from the planktonic larval stage to the attached juvenile. These include: the shell protein, conchiolin; the digestive enzyme, aryl sulfatase; and a novel serine protease. For each of these three, evidence for a multi-gene family has been obtained; strong evidence for gene-switching at metamorphosis thus far has been obtained for two of the three.

Our most significant advance in this area this year has been in the molecular cloning and analysis of cDNAs corresponding to the novel serine protease from the *Haliotis* intestine. This work required the development of several new methods which we believe will prove widely useful in molecular studies of many marine organisms. To isolate intact, full-length mRNAs and rRNAs from abalone tissues, we found it necessary to use guanidinium isothiocyanate for homogenization, followed rapidly by extraction with hot phenol. This method for inactivating ribonucleases has made possible the detection of an unusually large rRNA molecule; this is ca. 1 kilobase longer than the 28s molecule of vertebrates, and appears hyper-sensitive to cleavage near its center. The 18s rRNA species is more highly conserved. To our knowledge, this is the first detection of such a large rRNA from any mollusc; we are preparing a manuscript reporting this finding, and the general method which made it possible.

cDNA was constructed from the principal poly A^+-RNA purified from abalone intestine; the cDNA was purified, cloned in lambda gt-10, and sequenced by the Sanger dideoxy method. Presence of the transcript was found to be tissue-specific (by Northern analyses). Search of the NBRF protein data bank revealed it to encode a unique serine protease.

Recent advances in optimization of cDNA-synthetic reactions permitted detection of the full-length product in dried alkaline-agarose gels by autoradiography. This species was not detected by ethidium bromide fluorescence of dye-RNA complexes in formaldehyde RNA gels. Thus, analysis of full-length cDNA products in this manner from multiple tissues provides a general method for isolating tissue-specific cDNAs and studying gene expression in an organism whose gene products are poorly characterized. This powerful method is the basis of another manuscript in preparation.

The predicted structure of the substrate binding domain of the abalone protease resembles that of vertebrate elastase II enzymes, which preferetially hydrolyze peptide bonds at the carboxyl terminus of large hydrophobic residues such as leucine and phenylalanine. Elastase II is a hybrid of elastase I and chymotrypsin both in specificity and in the shape of the substrate binding pocket. Elastase I accommodates only small hydrophobic residues, while chymotrypsin binds large unbranched hydrophobic side chains. Elastase II will recognize both classes of amino acid substrates. The substrate-binding pocket of elastase I is bordered by the bulky pair val 216 and thr 226, that of chymotrypsin by the smallest pair gly 216 and gly 226, while elastase II contains the intermediate gly 216 and thr 226 pair. The abalone protease pocket is similarly rimmed with gly 216 and ser 226. Three additional invertebrate serine protease cDNAs with elastase II type substrate binding pockets also recently have been cloned. One encodes a protease secreted by *Schistosoma* cercariae to hydrolyze connective tissue; this was cloned through a collaboration of groups at UCSF and the Naval Biosciences Laboratory, Berkeley. The two others are derived from a family of serine protease genes in *Drosophila*, which, like the abalone gene(s), are expressed abundantly and specifically in the gut.

Analysis of the molluscan intestinal protease specificity using synthetic substrates confirmed the predictions made from the cDNA sequence analysis. Analyses of the sequence also predict a unique

(asparagine-isoleucine) site and mechanism for proteolytic autoactivation of the protease zymogen. This prediction is now being tested.

WORK PLAN (for Year 3):

Objective 1: Chemosensory receptors and signal transducers:

Our long-term objective here is a complete understanding at the molecular level of the mechanisms by which chemosensory signals, receptors and signal transducers regulate attachment and metamorphosis of marine invertebrate larvae. Our immediate objectives for the next year, building on our recent success in the in vitro resolution and purification of the receptors and transducers, are two-fold: (A) To determine the nucleotide and protein sequences corresponding to the receptors and signal transducers we have labeled and purified; and (B) to pursue the purification and functional reconstitution of the receptors and signal transducers as the best means to analyze the mechanisms of their actions in vitro.

Our strategies for gene cloning include: (1) heterologous probing and PCR-amplification of those sequences known to be highly conserved in the G proteins, calmodulins, and protein kinases, using at first the mRNA we already have purified from the isolated chemosensory cilia; (2) limited N-terminal protein sequence analysis of the labeled and purified G protein and phosphorylated proteins (the 25 kD target of PKA, and the 130 kD target of PKC), and use of these sequences to construct homologous oligonucleotide probes; and (3) covalent cross-linking of ligands to the ciliary chemosensory receptors, followed by purification, sequence analysis, and probe-construction as above, for the lysine and GABA receptors themselves. Success in the gene cloning and analysis will enable us to determine the structures and functions of the receptors and signal-transduction proteins; their mechanisms of interaction; and the mechanisms by which the genes for these components are regulated.

High priority among these objectives is the sequence analysis of the 25 kD and 130 kD proteins phosphorylated by PKA and PKC under the control of the GABA receptor and lysine receptor, respectively. These target proteins are essential and as yet poorly understood links in the transduction sequences by which chemical information from the environment regulates metamorphosis of the larvae. One hypotheses to be explored by the search of protein sequence data banks is that one or more of these phosphorylated proteins may be components of the membrane ion channels we have shown to be regulated by the chemosensory receptors.

Results of these studies will be significant in advancing our understanding of the basic mechanisms of chemical signal recognition, signal transduction, and regulation, not only in larval metamorphosis, but in other sensory, neuronal and developmental systems as well.

Objective 2: Mechanism of morphogenetic activation of specific gene expression in the developmentally arrested larva:

Our major objective is to further characterize the cloned protease genes and their protein products, and to use the cloned sequences as probes for developmental activation of differential gene expression, gene switching, processing and/or rearrangement, during the chemically induced metamorphosis and start of post-metamorphic growth.

Gene activation (transcription) will be monitored by quantitative dot-blot and Northern hybridization assays as a function of the induction of metamorphosis. The extent of the gene family, and DNA and mRNA processing or rearrangement, will be determined by hybridization in conjunction with genomic mapping. Toward this end, we will cast isolated abalone sperm nuclei in agarose plugs, for release of the DNA and digestion with the two known eight base-specific restriction enzymes, *Not* I

and *Sfi* I. These enzymes should fragment the two-million kilobase genome into appropriate sizes for FIGE gel analysis and subsequent Southern hybridization with the cDNA probe. The extent of linkage of these multiple hybridizing fragments can then be determined. Many members of the protease gene families, including those in *Drosophila*, are tightly linked in tandem.

For purification and characterization of the protein product, two recombinant abalone proteases will be expressed in *E. coli*, purified, and characterized. These have been produced by fusion of the 5' end of the abalone protease cDNA to two different bacterial genes. One is a simple fusion to the beta-galactosidase alpha-fragment gene on the modified pUC sequencing plasmid. Maintenance of an episome bearing the lac i^q gene is necessary for induction of the fusion protein from this extremely high copy number plasmid. The second construct is a fusion to the *S. aureus* protein A gene, which has been placed under the control of the lambda promoter, P_r. This fusion protein is expressed in a lambda c1857 lysogen upon a temperature shift from 30°C to 42°C. We will attempt first to detect these fusion proteases using sensitive new synthetic (fluorigenic) substrates. These procedures should make it possible to efficiently produce and purify the novel serine proteases encoded by the abalone genes we have discovered. This, in turn, will facilitate our molecular characterization of the protein product.

INVENTIONS (Year 2):

The molluscan protease gene sequences may be patentable. The enzymes they encode have a unique "hybrid" substrate-specificity.

PUBLICATIONS AND REPORTS (Year 2):

Manuscripts Enclosed:

1. Cariolou, M.A. and D.E. Morse. 1988. Purification and characterization of calcium-binding conchiolin shell peptides from the mollusc *Haliotis rufescens*, as a function of development. J. Comp. Physiol. B. 157:717-729.

2. Morse, D.E. 1988. Trigger and amplifier pathways: Sensory receptors, transducers and molecular mechanisms controlling larval settlement, adhesion and metamorphosis in response to environmental chemical signals. In: Marine Biodeterioration, ed. by M.-F. Thompson, R. Sarojini and R. Nagabhushanam. Oxford Press, Delhi, pp. 453-462.

3. Morse, A.N.C. 1988. The role of algal metabolites in the recruitment process. In: Marine Biodeterioration, ed. by M.-F. Thompson, R. Sarojini and R. Nagabhushanam. Oxford Press, Delhi, pp. 463-473.

4. Morse, D.E. and A.N.C. Morse. 1988. Learning from larvae: Chemical signals and molecular mechanisms controlling reproduction and metamorphosis. Oceanus 31(3):37-43.

5. Morse, D.E. 1989. Recent progress in larval settlement and metamorphosis: Closing the gaps between molecular biology and ecology. Bulletin Marine Science (in press).

6. Morse, D.E. 1989. Morphogens, signal molecules, and other non-toxic bioactive substances that play a role in structuring interactions and distributions in the marine environment. In: Bioactive Substances from Marine Organisms, ed. by M.-F. Thompson and R. Nagabhushanam, Oxford Press, Delhi (in press).

7. Morse, A.N.C. 1989. GABA-mimetic peptides from marine algae and bacteria as potential diagnostic and therapeutic agents. In: Bioactive Substances from Marine Organisms, ed. by M.-F. Thompson and R. Nagabhushanam, Oxford Press, Delhi (in press).

8. Markell, D.E. and D.E. Morse. 1989. Regulation of expression of GABA receptors on developing *Haliotis rufescens* larvae: Computer-assisted analysis. Developmental Biology (submitted).

In Preparation:

1. Baxter, G. and D.E. Morse. 1989. Chemosensory lysine receptor controls G protein and protein kinase C in cilia purified from molluscan larvae in vitro. For: J. Biological Chemistry (in preparation).

2. Groppe, J and D.E. Morse. 1989. Detection of high molecular weight rRNA from the marine mollusc, *Haliotis rufescens*. For: Nucleic Acids Research (in preparation).

3. Groppe, J. and D.E. Morse. 1989. A novel method for cloning of abundant, tissue-specific cDNAs. For: J. Biological Chemistry (in preparation).

4. Groppe, J. and D.E. Morse. 1989. Molecular cloning of novel serine protease cDNAs from abalone. For: Proc. Natl. Acad. Sci. (in preparation).

5. Spaulding, D. and D.E. Morse. 1989. Arylsulfatases from larval and adult *Haliotis rufescens*: evidence for gene-switching during development. For: J. Comp. Physiol. B. (in preparation).

6. Morse, D.E. 1989. Molecular mechanisms controlling metamorphosis and recruitment in abalone larvae. For: Abalone Biology, Fisheries and Cultivation, ed. by M. Tegner and S. Shepherd (in preparation).

7. Morse, D.E. and A.N.C. Morse. 1989. What are planktonic larvae "waiting" for? A molecular view of the morphogens, receptors, and signal transducers controlling site-specific metamorphosis of animals in the ocean. Invited article for: American Scientist (in preparation).

Abstracts and Posters:

1. Morse, D.E. 1988. Chemical signals control site-specific settlement and metamorphosis of planktonic larvae: Characterization of the signals, receptors, transduction and regulatory mechanisms. (Abstract) Proc. Intl. Chemical Ecology Symposium., Athens, Georgia.

2. Morse, D.E. 1988. Molecular mechanisms controlling larval settlement and metamorphosis: A focus of the molecular marine biology program at the University of California, Santa Barbara. (Abstract) Proc. First. Intl. Symp. Mar. Molec. Biol., Center of Marine Biotechnology, University of Maryland.

3. Morse, D.E., A.N.C. Morse, G. Baxter and R.A. Jensen. 1988. Recent progress in the characterization of chemical signals, larval receptors, signal transducers and amplifiers controlling the settlement and metamorphosis of marine invertebrate larvae. (Abstract) Proc. Symp. Amer. Zool., San Francisco, CA.

4. Groppe, J.C. and D.E. Morse. 1989. Cloning and identification of a molluscan serine protease cDNA. (Poster) Arrowhead Genetics Conference, Arrowhead, CA.

5. Morse, D.E. 1989. Morphogens, signal molecules, and other non-toxic bioactive substances that play a role in structuring interactions and distributions in the marine environment. (Abstract) Proc. Intl. Symp. Bioactive Substances from Marine Organisms., Goa, India.

6. Morse, A.N.C. 1989. GABA-mimetic peptides from marine algae and bacteria as potential diagnostic and therapeutic agents. (Abstract) Proc. Intl. Symp. Bioactive Substances from Marine Organisms., Goa, India.

7. Roell, M.K. and D.E. Morse. 1989. A rapid method for isolation of chloroplast DNA from the red alga, *Polysiphonia boldyii*, and hybridization with a cyanobacterial phycoerythrin gene. (Poster and Abstract) Northwest Algal Symposium, Seattle, WA.

8. Groppe, J.C. and D.E. Morse. 1989. Molecular cloning of novel serine protease cDNAs from abalone. (Abstract) Proc. First Intl. Symp. Marine Biotechnology, Tokyo.

9. Roell, M.K. and D.E. Morse. 1989. A rapid method for isolation and fractionation of nucleic acids from the red alga, *Polysiphonia boldyii*. (Poster and Abstract) Intl. Symp. Amer. Soc. Phycology, Toronto.

Reports:

1. Annual Report, June 1988, to ONR Molecular Biology Program and Distribution List.

2. Annual Report, June 1989, to ONR Molecular Biology Program and Distribution List.

GRADUATE STUDENT TRAINING ACTIVITIES:

Graduate Student Trainees	3	(1 thesis completed)
Women or Minorities	1	
Non-citizens	0	

AWARDS/FELLOWSHIPS:

One graduate student awarded University of California Marine Biotechnology Traineeship.

One graduate student awarded University of California Institute of Marine Resources Dissertation Year Fellowship.

OTHER APPOINTMENTS (PI):

Chairman, NSF Taskforce on the Transfer of Molecular Biology and Biotechnology to Ocean Sciences.

Chairman, Marine Biotechnology Center, University of California at Santa Barbara.

U.S. Co-chairman, Indo-U.S. International Symposium on Bioactive Substances from Marine organisms. Goa, India. February 1989.